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Affinity Chromatographic Method for the Extraction of Ocytocin from Human and Rat Plasma

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Summary: A procedure based on affinity chromatography for the extraction of ocytocin is described. Ocytocin antibody, covalently attached to a magnetizable iron suspension, retains its ability to bind ocytocin from blood plasma. The conjugate can be reused several times. Recovery of ocytocin was 60–70%, and the detection limit for the extraction assay was about 1 pg per tube. The specificity of the extraction method was characterized by HPLC/RIA on specimens extracted from blood plasma. The extracted material was found to be chomatographically identical with the reference ocytocin preparation. Comparison of RIA data for the same samples extracted by immunoextraction (y) and adsorption (x) showed a linear regression of $y = 0.83\ x + 2.12$, $r = 0.81$, $n = 17$.

Introduction

The neurohypophysial hormone, ocytocin, has various functions. It acts as a peripheral hormone, as a neuropeptide in the brain, and as a paracrine substance in several tissues. As a hormone, it regulates parturition and lactation; as a neuropeptide, it participates in memory processes, maternal and sexual behaviour, and the development of tolerance and addiction to narcotic drugs; as a paracrine factor, it plays a role in various peripheral organs (e. g. the ovary). Its biological importance stimulated various groups to develop methods for measurement of the hormone. Its basal circulating levels in the blood are low. Most of the RIA determinations involve extraction techniques, prior to the assay of ocytocin in the plasma (1–5). Extraction is necessary to eliminate non-specific interfering substances in the plasma, but the procedures are time-consuming and may involve significant and variable losses of ocytocin.

Our aim was to study the ocytocin extraction based on affinity chromatography with magnetizable iron particles. For this purpose, the ocytocin antibody was covalently attached to the surface of an iron suspension, and its ability to bind and extract ocytocin from human and rat plasma was examined. Additionally, the reusability of the iron-antibody conjugate was investigated.

Materials and Methods

Human blood was collected in EDTA-containing tubes at 8–9 a. m. from the antecubital vein of healthy subjects in the supine position. After centrifugation for 10 min at 1500 *g* and 4 °C, the plasma was collected in polystyrene tubes and stored at −70 °C until extraction.

Adult male Wistar rats weighing 180–230 g were used. Blood samples were obtained from the trunks of decapitated rats between 8 and 10 a. m., collected in polystyrene tubes containing EDTA in isotonic saline, maintained on an ice-bath, subsequently centrifuged at 2000 *g* for 10 min at 4 °C and stored at −70 °C until extraction.

Materials

The sources of materials were as follows: synthetic ocytocin (Bachem, Bubendorf, Switzerland), N-terminal Biomag magnetic particles (Advanced Magnetics, Inc., Cambridge, MA), bis-succinimidyl suberate (Serva, Heidelberg, Germany), trifluoroacetic acid (Fluka, Germany), methanol (HPLC grade), Veronal-Na (Merck, Germany), bovine serum albumin, *L*-cystine (Sigma, St. Louis, USA). All other chemicals were of analytical grade.
The RIA buffer consisted of 20 mmol of Veronal-Na, 10 mmol of EDTA-Na_2, 0.14 mol of NaCl, 5 g of bovine serum albumin and 0.01 g of *L*-cystine per litre, pH = 8.

Antibody preparation and purification

The ocytocin antiserum against ocytocin-ε-aminocaprilyl bovine thyroglobulin was produced in rabbit (6).

The ocytocin antibody was purified before use in the coupling procedure. The IgG fraction of the native antiserum was precipitated by dropwise addition of an equal volume of 360 g/l sodium sulphate. After centrifugation, the precipitate was washed with 18 g/l sodium sulphate. The pellet was dissolved in 0.03 mol/l HCl, and 0.03 mol/l NaOH was added dropwise until opalescence and precipitation occurred. The mixture was centrifuged at 2000 *g* for 15 min at 4 °C, and the resulting pellet was dissolved in isotonic saline and dialysed against distilled water for 2 days at 4 °C. The dialysed fraction was lyophilized.

Methods

Antibody coupling to magnetic particles

The coupling procedure was performed according to the bis-succinimidyl suberate active ester method (7). Lyophilized (10–20 mg) ocytocin antibody was coupled to 1 ml of magnetic suspension. The resulting antibody-coated particles were washed three to four times with 0.03 mol/l HCl, followed by 0.03 mol/l NaOH, then stored in assay buffer containing 0.2 g/l NaN_3 (sodium azide) as preservative. After immunoextraction, the sedimented magnetic iron conjugate was washed twice with 500 µl of RIA buffer, made up with assay buffer (containing 0.2 g/l sodium azide) to the final volume (representing a two or fourfold dilution of the conjugate), then stored at 4 °C.

Procedures

Immunoextraction

One ml of human plasma was mixed with 200 µl of magnetic suspension diluted with assay buffer, and rotated end-over-end for 3 hours at 4 °C. To separate the particle-bound fraction, the tubes were placed on a magnetic rack, the particles were sedimented and the supernatant was discarded. The magnetic suspension was washed twice with 500 µl of assay buffer. The ocytocin was then eluted from the magnetic iron conjugate with 400 µl of methanol/water (80 + 20, by vol.) containing 1 ml/l trifluoroacetic acid. An aliquot (300 µl) of the supernatant was dried under a nitrogen stream. The residue was dissolved in 225 µl of RIA buffer and analysed in duplicate (6).

Standard concentrations of ocytocin (0.8–100 pg per tube) were prepared in assay buffer, or in "ocytocin-free plasma" and immunoextracted. Peptide-free plasma was made from pooled plasma samples, pretreated with activated charcoal, centrifuged, and purified on Sep-Pak cartridge.

Adsorption method

The method was based on that described earlier (8). Lichroprep Si60 (20 mg), distilled water (100 µl) and 1 mol/l HCl (400 µl) were added to 2 ml of blood plasma in polypropylene tubes. The mixture was agitated for 30 min then centrifuged. The supernatant was discarded, the pellet washed twice with distilled water, and the immunoreactive material eluted with acetone/water (60 + 40, by vol.), evaporated and assayed.

Radioimmunoassay

A relatively sensitive in-house RIA was set up (6).

The ocytocin antibody affinity constant was 3.7×10^9 mol/l. Cross reaction with related peptides: ocytocin 100, vasotocin 0.11, ocypressin 92.7, $ocytocin_{1-8}$ 3.26, arginine-vasopressin 0.11%, pressionic acid, melanostatin and $ocytocin_{4-8}$ less than 0.05%. [^{125}I]Ocytocin was prepared by the method of *Hunter & Greenwood* (9) and purified by two-step reverse-phase chromatography (10). The specific radioactivity was about 70.3 TBq/mmol.

For the RIA, 50 µl of ocytocin antibody (at a final dilution of 1 : 70 000) were added to 100 µl of sample or standard, then incubated for 24 h at 4 °C, followed by addition of iodine-labelled ocytocin (10 000 counts/min in 50 µl). After incubation for a further 24 h, the bound and free conjugates were separated by adding 50 µl of horse serum and 500 µl of polyethyleneglycol in water, 300 g/l. After centrifugation at 1500 *g* for 15 minutes, the radioactivity of the pellet was measured on a Wizard automatic gamma counter (Wallac Oy, Finland), with subsequent calculation via the Multicalc program (Wallac Oy, Finland).

High performance liquid chromatography

The procedure was performed as described earlier (11). Eluates of 39-39 immunoextracted human or rat plasma samples were pooled and evaporated. The residues were dissolved in methanol/water (30 + 70, by vol.) containing 1 ml/l trifluoroacetic acid. A Knauer HPLC system with gradient elution was used to characterize the ocytocin-like immunoreactivity. Aliquots of the dissolved samples were loaded onto a Si-100-S RP-C18 column (5 µm, 260 × 4.6 mm; BST, Budapest, Hungary) and eluted in a gradient system of aqueous methanol, volume fractions 0.3–0.6 containing 1 ml/l trifluoroacetic acid. To avoid contamination, specimens were run in the sequence: blank, pooled human or rat samples, and finally standard ocytocin (0.5 ng) (12). The flow rate was 1.0 ml/min.

The eluted HPLC fractions were collected at 1-min intervals for up to 50 min and subjected to RIA (figs. 1a, b).

Results

The purified ocytocin antibody was coupled to N-terminal magnetizable particles by the active ester method. To exclude interference from endogenous ocytocin, the conjugate was purified. Pretreatment of the antibody and repeated washing of the iron-antibody conjugate led to an increase in the binding and a reduction of the non-specific binding to 3%.

The ocytocin binding capacity of the antibody-coupled particles was determined by adding iodine-labelled ocytocin to serial dilutions of particle suspension. The binding of the ocytocin tracer for the two- or fourfold diluted suspension was 85 and 78%, respectively. The standard curve was corrected for the ocytocin-free plasma, to eliminate extraction losses and procedural errors.

The efficiency of the immunoextraction was determined by adding different amounts of ocytocin to the rat plasma pool. The following recoveries were found: 50 ng/l 61.7 ± 6.4%, 25 ng/l 71.2 ± 5.2% and 12.5 ng/l 74.2 ± 7.6%, respectively, (x ± SEM, n = 5).

Intra- and inter-assay coefficients of variation determined on pooled rat plasma specimens at a concentration of 12.1 ± 0.8 ng/l were 7.9 and 13.9% (n = 6), and at 46.7 ± 1.8 ng/l 5.3 and 10.1% (n = 6), respectively. The detection limit of the assay after immunoextraction was 1 pg per tube.

The basal concentration in rat plasma was found to be 15.28 ng/l ± 0.84 SEM, n = 6.

The residual extracted suspension was washed with RIA buffer, made up to final volume and stored. Repeated use of the conjugate was investigated. For three successive immunoextractions of plasma the following ocytocin values were obtained: 15.9 ± 4.0, 17.2 ± 3.4 and 14.6 ± 3.0 ng/l, n = 6.

In HPLC, a major proportion of the ocytocin immunoreactivity in both the human and rat plasma samples migrated together with the reference ocytocin preparation (figs. 1a, b).

To assess the accuracy of the immunoextraction, the results from immunoextracted human samples were compared with those after adsorption extraction.

Regression of the results gave the equation y = 0.83 x + 2.12, r = 0.81, n = 17, SE slope 0.15, Yse 1.28.

	After adsorption extraction
females	3.9 ± 0.6 SE, n. s., n = 11
males	4.1 ± 0.9 SE, n. s., n = 6
	After immunoextraction
females	5.7 ± 0.6 SE, n. s., n = 11
males	5.2 ± 0.6 SE, n. s., n = 6

(two way Anova, followed by *Scheffe*'s rank test)

Discussion

A sensitive RIA is dependent on the production of a highly specific and high-affinity antiserum with a radiolabelled tracer of adequate specific activity. These properties are necessary for direct measurement without prior extraction (13, 14). Many techniques have been

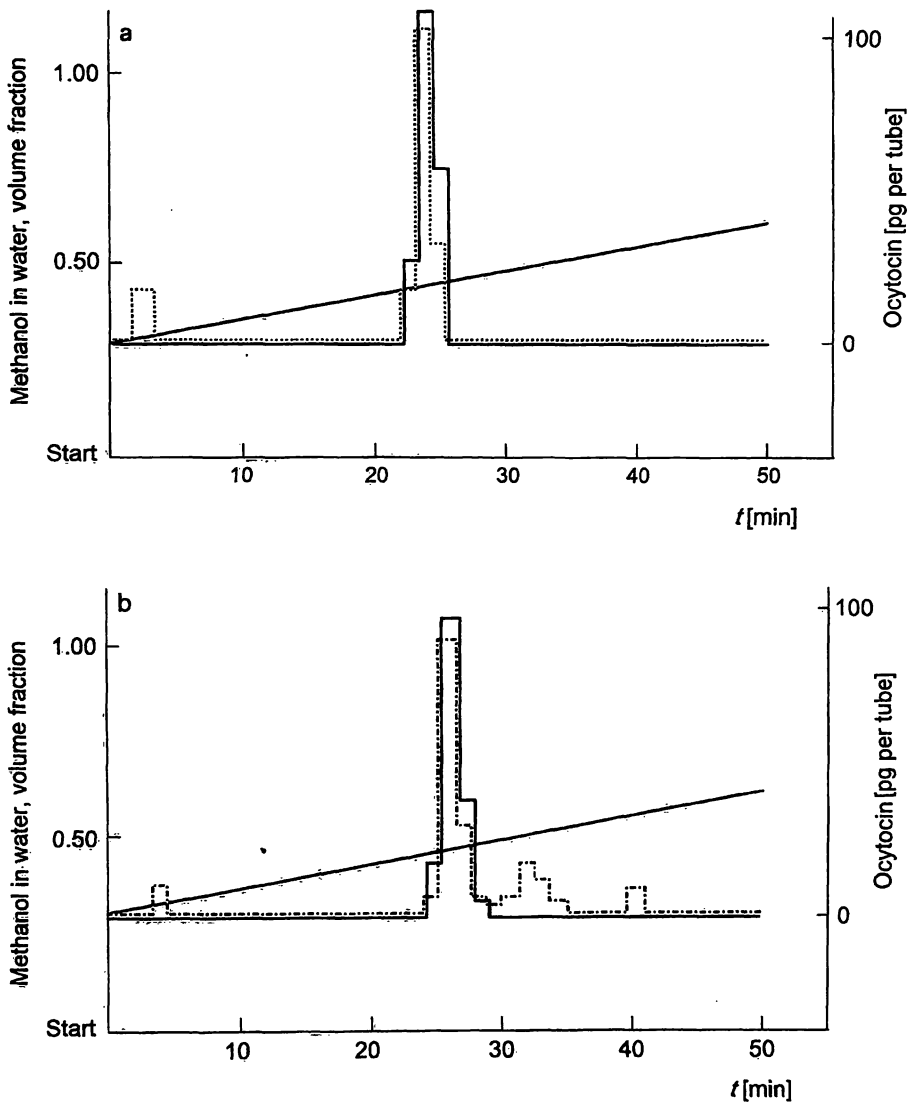

Fig. 1a, b Elution profile of immunoextracted human and rat plasma samples. The elution was performed with a gradient system of aqueous methanol containing 1 ml/l trifluoroacetic acid, and fractions were radioimmunoassayed for ocytocin. The immunoreactive material was eluted at the same position as the reference ocytocin (—), in a) human (···) and b) rat (-·-·-) plasma extracts.

described for the extraction and concentration of ocytocin from plasma. Most methods are based on simple physicochemical techniques. An acid-ethanol mixture has been used to precipitate proteins from blood samples (15), while other, similar procedures involve the use of acetone (16, 17). Another type of extraction procedure involves the adsorption of ocytocin onto particulate materials such as *Fuller*'s earth or glass beads (4, 18, 19). The main disadvantage of these methods is that the procedures are non-specific, often work better from acidified plasma and display mediocre recovery rates. The Sep-Pak mini cartridge (20, 21), and an affinity extraction method (22) give better results.

The HPLC results demonstrated that the immunoreactive ocytocin is physicochemically identical to the corresponding synthetic molecule. Slightly higher plasma levels of ocytocin measured in the present study in comparison with the adsorption method or with other reports could have been due to certain methodological variations.

Repeated use of the washed antibody-iron conjugate appears promising. It seems that the recovery after several washing or elution steps is reproducible. The weakly bound peptides or proteins can readily be removed.

In conclusion, we have set up a new and specific method based on affinity chromatography for the extraction of ocytocin from blood plasma samples.

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